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PRINCIPAL INVESTIGATOR: Josiah Ochieng, Ph.D.

CONTRACTING ORGANIZATION: Meharry Medical College
Nashville, Tennessee 37208

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Josiah Ochieng, Ph.D.

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Meharry Medical College
Nashville, Tennessee 37208

E-Mail:jochieng@mmc.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Our overall objective is to delineate the role of galectin-3 in the interactions between breast carcinoma cells and elastin. Therefore the scope of this proposal is to prove to ourselves and convince the scientific community that expression of galectin-3 by breast cancer cells is not only important for growth, but also the interaction of these cells with elastin, an interaction which has significant consequences when one considers the propagation of metastatic cells in elastin rich tissues. We have at this point demonstrated by transfecting tumorigenic breast epithelial cells with antisense oligos to galectin-3 that indeed galectin-3 is critical for the interaction of these cells elastin. We have also demonstrated that fetuin, a serum glycoprotein is a necessary for these tumor cells to release galectin-3 which is then used to ligate the cells to elastin. We have also narrowed the motif in elastin which is involved to the VGVAPG chemotactic domain. As for the galectin-3 the interacting domain encompasses the carbohydrate recognition domain. In the next year we hope to pinpoint the peptide domain(s) in galectin-3 which are responsible for galectin-3/elastin interaction.

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Progress Report
Josiah Ochieng

INTRODUCTION:

Galectin-3, a member of beta-galactoside binding proteins has been implicated in the cell to extracellular matrix interactions (for review see 1). We have demonstrated that galectin-3 regulates not only the adhesion of cells to extracellular matrix proteins mediated by integrins (1,2) but also interaction of cells with elastin, a member of the extracellular matrix proteins (3). The long term goal of this project is to demonstrate that the galectin-3 mediated cell/elastin interaction has direct consequences in the interactions of cells with elastin rich tissues and in the proliferative potential of the breast carcinoma cells. To date very little attention is given to the interaction of cells with elastin particularly in elastin rich tissues such as the lungs. The growth of metastatic nodules in the lungs may ultimately depend on cell to elastin interactions as our studies demonstrate.

BODY:

The following are the research activities that have been carried out in the laboratory since October of 2000. In the past one year our goal was to complete specific aim # 1 through Task # 2. Specifically we set out to; a) demonstrate the consequences of down-regulating the expression of galectin-3 by galectin-3 antisense oligos in breast carcinoma cell/elastin interactions (months 12-24), and b) to establish the contribution of cell surface galectin-3 in cell/elastin interactions.

Task 2 . To establish the contribution of cell surface galectin-3 to cell/elastin interactions (months 8-24)

To establish a role for galectin-3 in cell to elastin adhesion, we coated wells of a microtiter plate with soluble elastin (either solubilized by hydrazine or hot oxalic) at a concentration of 200 ug/well. Non-specific binding sites in the wells were blocked by 2% BSA and both BT-549 (galectin-3 null expressing cells) and 11-9-1-4 (BT-549 transfected with galectin-3 gene) were added to the wells in the absence and presence of fetuin and in the absence of divalent ions. The cells were allowed to bind to the wells overnight after which non-adherent cells were washed off and the degree of adhesion determined. It was evident that galectin-3 expressing cells, 11-9-1-4 adhered much better to the elastin coated wells compared to BT-549. The adhesion of 11-9-1-4 was further improved in the presence of fetuin (Figure 1). We have demonstrated that fetuin has the ability to induce the rapid secretion of galectin-3 from the cells (4). We have repeated these experiments over 10 times under different conditions and in all these cases, we show that adhesion of cells to elastin require galectin-3. Adhesion is improved in the presence of exogenously added recombinant galectin-3. The data suggest that galectin-3 in these cases ligates the cells to elastin via its cell surface ligands.

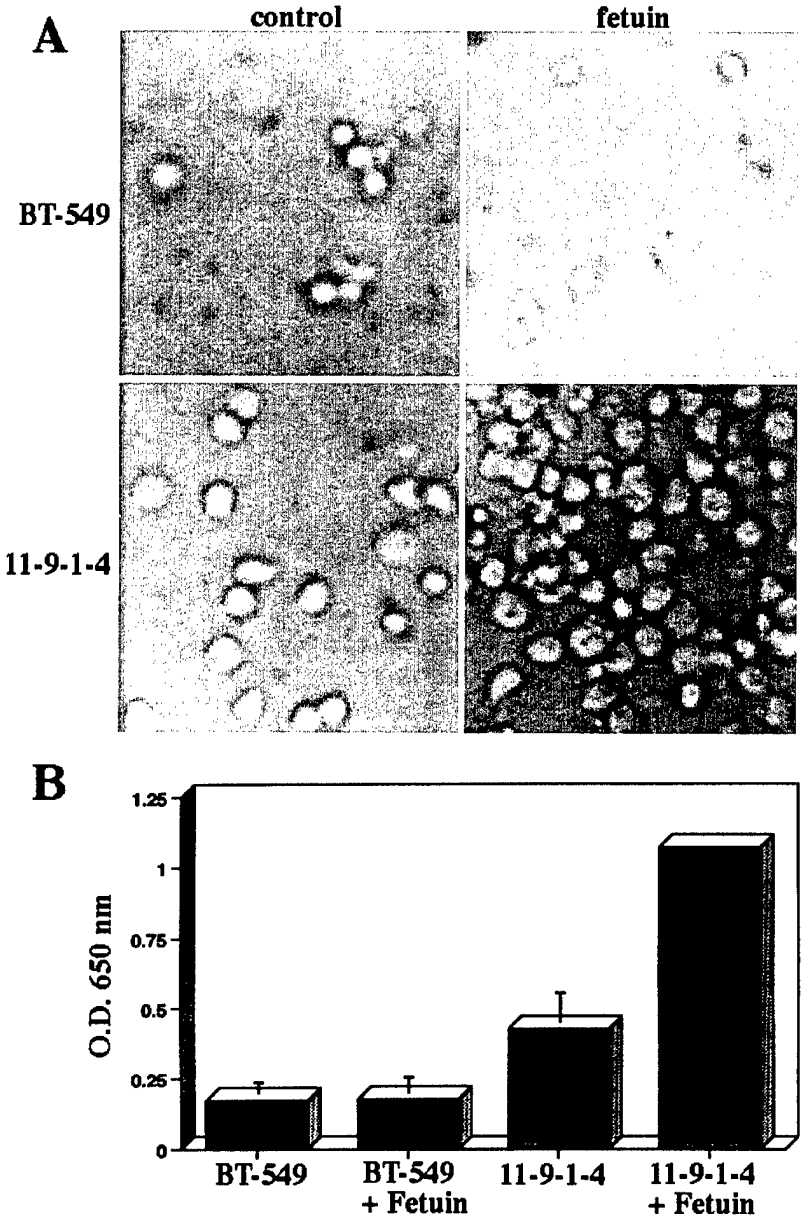

Figure 1. Ligation of breast carcinoma cells to elastin. In A, the wells of a microtiter plate were coated with hydrazine solubilized elastin. After blocking non-specific sites with 2% BSA, the cells (5 X 10^4 cells/well) were plated and allowed to adhere to wells in serumless DMEM/F12 medium without divalent ions and without (control) or with 0.25% fetuin. Non- adherent cells were washed and adherent cells photographed. In B, the adherent cells were fixed in methanol and cell number determined.

Task 2. Down-regulation of galectin-3 expression and cell/elastin interactions.

In this task we transfected the breast carcinoma cell line MDA-MB-231 with galectin-3 antisense oligos as well as scrambled antisense control as described in the grant, by the lipofectin protocol. We chose this cell line instead of the MDA-MB-435 because its interaction with fetuin is much better compared to the other breast carcinoma cell lines which produce galectin-3. The success of transfection was determined by determining the level of galectin-3 in the cell lysates by western blotting. We were not able to completely block galectin-3 expression by antisense oligos. It is possible that in those cells that galectin-3 expression was completely eliminated, cell viability was lost and so could not be used in the adhesion studies. We however took the cells in which expression was reduced by approximately 40% and allowed these to adhere to elastin coated wells and the adhesion potential compared to those of cells transfected with the scrambled antisense sequence as well as wild type controls. It was gratifying to observe that cells transfected with the galectin-3 antisense oligos demonstrated a significantly reduced adhesion to elastin. Their adhesion was approximately 70% of the adhesion seen in scrambled antisense transfectants and wild type controls (Figure 2).

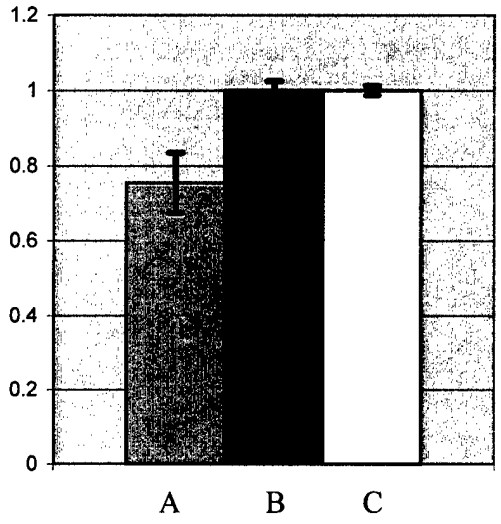

Figure 2. **Adhesion of Breast Carcinoma cells to Elastin**. The wells of a microtiter plate were coated with soluble alpha human lung elastin (2 mg/ml) and none-specific sites blocked with 1% BSA. The cells (MDA-MB-231) transfected with galectin-3 antisense oligos (A), scrambled oligos (B) and wild type controls (C) were added to the wells in complete DMEM medium. The wells were washed and the adherent cells determined by methylene blue method (5).

Even though the reduced interaction of galectin-3 antisense transfected cells with elastin was significant, it was not as substantial as we expected. It is possible that other galectins such as galectin-4 or 8 may also be involved in the ligation process. Apart from the studies earmarked for this period, we also continued to map the domains in both galectin-3 and elastin that are critical for interactions. We have made significant progress in task # 1. This task initially gave us a number of problems which we had not anticipated. However we have now established (and are getting ready to prepare the manuscript) that the minimal amino acid motif in elastin required for galectin-3 interaction is the VGVAPG, or the chemotactic domain of elastin. We did ligand blotting experiments to

establish this fact. Briefly we transferred recombinant galectin-3 to immobilon membrane and then incubated the membrane with the VGVAPG peptide followed by monoclonal antibodies made specifically to this peptide. In these studies we show that not only do the peptides bind specifically to galectin-3 but that the 22 kDa peptide appear to bind the peptide more strongly than the whole molecule (Figure 3).

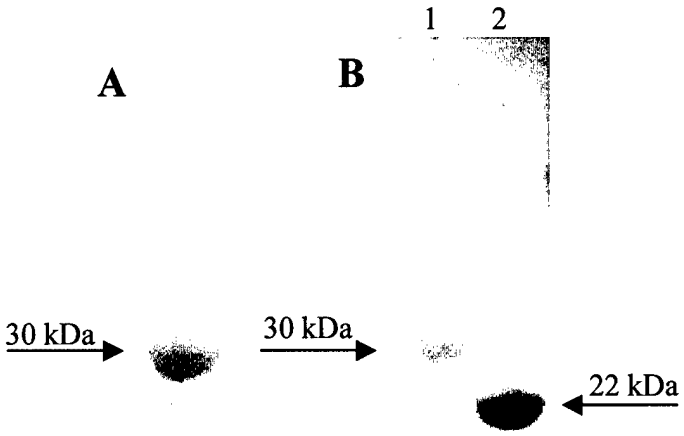

Figure 3. Binding of elastin peptide (VGVAPG) to Galectin-3. Galectin-3 was separated by SDS-PAGE, transferred to PVDF membrane incubated with elastin peptide. Bound peptide was detected by monoclonal anti-elastin antibodies and secondary anti-mouse HRP conjugated antibodies. In **A** 0.5 mg/ml concentration of elastin peptide allowed to detect the major band of galectin-3 at 30 kDa. In **B**, galectin-3 before **(lane 1)** and after **(lane 2)** cleavage by gelatinase MMP-2, was incubated with elastin peptide at concentration 0.25 mg/ml. Reaction with uncleaved galectin-3 is weak **(lane 1)** but very intensive after gelatinase treatment **(lane 2)** at 22 kDa.

These studies are in contrast to the studies which we reported last year in our progress report. This is because we had actually not standardized or optimized the binding protocols. We are currently repeating these experiments using the biocore method which will be able to determine for us the binding parameters including the on and off rates of binding.

Future Directions. We are now ready to begin Task 3 for the next 12 months. We have done some preliminary studies that are very encouraging . Basically we have shown that galectin-3 and the chemotactic peptide of elastin can dramatically alter the phosphorylation status of a number of intracellular proteins. We hope to report these studies in the Era of Hope Conference which we will attend next year.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrating that the chemotactic domain is actually the domain which specifically bind to galectin-3 and that the metalloproteinase cleaved fragment of galectin-3 interacts more effectively with the elastin peptide
- The observation that fetuin is involved in the secretion of galectin-3 which in turn ligates the cells to elastin.

REPORTABLE OUTCOMES:

Manuscript published in Cancer Research.

CONCLUSION:

In conclusion we have now established that expression of galectin-3 is critical for the interaction of cells with elastin. This interaction is important if we are to really begin to address ways and means of controlling the dissemination of breast cancer to elastin rich tissues such as the lungs where the adhesion of the cells to the lungs may trigger a rapid growth rate as a result of novel signaling events. The studies strongly suggest that inhibition of elastin-galectin-3 interactions could be a very critical mechanism which can be exploited in the development of novel anti-metastatic drugs. We have recently acquired a galectin-3 phage library (containing fragments of galectin-3 epitopes), and will use this library to map the domains of galectin-3 that are involved in the in the galectin-3/elastin interaction. These are very exciting experiments which we hope will give us enough preliminary data to apply for an RO1 grant to invest more research dollars in this field. Once more the financial help from the DOD is highly appreciated by my laboratory. We plan to have two more manuscripts submitted for publication by the end of the next 12 months.

REFERENCES:

1) Hughes, R.C. (2001) Galectins as modulators of cell adhesion. Biochimie 83: 667-676
2) Ochieng, J., Leite-Browning, M.L., and Warfield, P. (1998) Regulation of cellular adhesion to extracellular matrix proteins by galectin-3 Biochem. Biophys. Res. Commun. 246: 788-791.
3) Ochieng, J., Warfield, P., Green-Jarvis B., and Fentie, I. (1999) Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. J Cell. Biochem. 75: 505-514.
4) Wen-Qin Zhu and Ochieng, J. (2001) Rapid release of intracellular galectin-3 from breast carcinoma cells by fetuin. Cancer Res. 61: 1869-1873.

5) Oliver, M.H., Harrison, N.K., Bishop, J.E., Cole, P.J., and Laurent G.J.A. (1989) A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell Science 92: 513-518.

[CANCER RESEARCH 61, 1869–1873, March 1, 2001]

Rapid Release of Intracellular Galectin-3 from Breast Carcinoma Cells by Fetuin[1]

Wen-Qin Zhu and Josiah Ochieng[2]

Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208

ABSTRACT

Galectin-3, a β-galactoside binding protein, plays a significant role in cell to extracellular matrix interactions. Despite its extracellular expression, the precise physiological mechanisms that trigger its release from the intracellular milieu have not been characterized. The present analyses were, therefore, done to identify the extracellular matrix proteins with propensity to induce the release of intracellular galectin-3 from breast carcinoma cells. Our studies demonstrate that fetuin, a serum glycoprotein that is abundant in the fetal serum, is capable of inducing the rapid release (~1 min) of intracellular galectin-3 from the cells. The mechanism by which galectin-3 is rapidly released appears to be novel and does not depend on changes in intracellular calcium levels. We also report that galectin-3-expressing breast carcinoma cells in serumless medium adhere and spread well on microtiter wells in the presence of fetuin and divalent ions in a carbohydrate-dependent manner. The data suggest that fetuin is a natural modulator of galectin-3 secretion/release and that the secreted galectin-3 modulates the activity of cell surface receptors for extracellular matrix proteins.

INTRODUCTION

Galectins are a growing family of carbohydrate-binding proteins that share affinity for β galactosides and significant sequence homology in their carbohydrate-binding domains (1–3). Galectin-3 is expressed in the nucleus, in the cytoplasm, and on the cell surface of most epithelial cells and can be secreted into the extracellular matrix (4). On the cell surface, galectin-3 plays critical roles in cell-cell or cell-extracellular matrix interactions. It has been shown to mediate homotypic aggregation that may be responsible for tumor emboli (5, 6). It has also been shown to be responsible for rapid adhesion of breast carcinoma cells to ECM[3] proteins such as collagen IV, laminin, and elastin (7, 8).

Galectin-3 like many other cytosolic proteins, such as thioredoxin (9), interleukin-1β (10), and acidic and basic fibroblast growth factor (11), can traverse the plasma membrane and yet lacks signal peptides necessary for secretion via the classical secretory pathway. The mechanisms by which galectin-3 and other proteins that lack signal peptides are secreted via the nonclassical pathway have yet to be elucidated. Galectin-3 may be concentrated in secretory vesicles that are concentrated in the membrane domains (12). It has been shown that NH_2-terminal domain of galectin-3 is critical for its secretion and is the driving force that localizes it in the secretory vesicles (13, 14, 15). How galectin-3 moves from these vesicles into the ECM or the mechanism that triggers the exocytosis of these vesicles is the gap in our current knowledge.

It has been demonstrated that whereas secretion of galectin-3 is normal in medium that contains serum, it is dramatically reduced in serumless medium (4). In the present study, we have exposed breast carcinoma cells to different ECM proteins in order to identify the ECM proteins likely to elicit the release/secretion of galectin-3. Our studies demonstrate that fetuin, the serum glycoprotein abundant in fetal blood, if added to serumless medium in concentrations similar to that in medium supplemented with 10% fetal bovine serum, is capable of releasing intracellular galectin-3 rapidly from breast carcinoma cells. Fetuin was previously shown to bind to various tumor cells and to induce cell aggregation by binding to lectin-like molecules (6). More recently, it was demonstrated that insect cells that express galectin-3 on their surfaces undergo homotypic aggregation in the presence of asialofetuin or fetuin (5). It is, therefore, possible that fetuin can interact with cell surface lectins and that this is sufficient signal to trigger the release of galectin-3 and possibly other members of the family. Galectin-3 is released rapidly from intracellular domains in a dose-dependent manner. Changes in intracellular calcium ion concentration do not influence the release that appears to be mediated by a novel mechanism. The data further suggest that the galectin-3 released by the cells is responsible for the rapid adhesion and spreading of breast carcinoma cells to the substrata.

MATERIALS AND METHODS

Human breast epithelial cell lines MDA-MB-435; BT-549; and 11-9-1-4, which is a galectin-3-transfected BT-549, were kindly donated to us by Dr. Avraham Raz, Karmanos Cancer Institute, Detroit, MI. All of the cell lines were cultured in DMEM/F12 (Sigma) supplemented with 100 μg/ml penicillin-streptomycin, 2.5 μg/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin, 10% heat-inactivated fetal bovine serum, 2 mM glutamine and nonessential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Human ECM was purchased from Collaborative Research and all of the other biochemicals from Sigma Chemical, unless otherwise stated.

Rapid Release of Galectin-3 from Cells. To assay for galectin-3 release, the cells were removed from the culture flasks by trypsinization and washed twice in serumless DMEM/F12 medium. After the last wash, the cells were left standing in suspension in the centrifuge tubes for at least 10 min. The cells were then counted using a hemocytometer and 500,000 cells in approximately 20 μl of serumless medium added to siliconized Eppendorf tubes containing 100 μl of serumless DMEM/F12 with the various additives. In the first experiment, the additives were: (*a*) fibronectin (2 mg/ml); (*b*) collagen IV (2 mg/ml); (*c*) human ECM (2 mg/ml); (*d*) TDG (100 mM); (*e*) lactose (100 mM); (*f*) 10% serum; and (*g*) 0.25% fetuin. The samples were incubated for 10 min at room temperature and centrifuged to pellet the cells, and the supernatant from each tube (20 μl) was assayed for galectin-3 by Western blot as described previously (6). The galectin-3 release assay was repeated with different doses of fetuin (0–1%) and with 0.25% fetuin to obtain a time-course of release.

The Source of Released Galectin-3. To demonstrate that the secreted fetuin is from intracellular domains and not from the cell surface, cell surface proteins were labeled with biotin, and the labeled proteins were then chased after treatment of the cells with fetuin. Briefly, cells in 75-cm^2 culture dishes were washed with serumless medium five times and then with PBS two times. The cells were then incubated with 2.6 mM NHS-Biotin (Biorad) for 30 min at room temperature with occasional swirling. The unreacted biotin was removed and the flasks washed thoroughly with PBS, and then trypsinized. Trypsin was inactivated by the addition of complete medium containing serum, and the cells were centrifuged. The cells were washed twice with serumless medium and then divided into two Eppendorf tubes (500,000 cells/tube) in 100 μl of serumless medium without (control) or with 0.25% fetuin. The cells were then incubated for 30 min at 37°C and centrifuged; 20 μl of the supernatant were taken from each tube (conditioned medium) and applied to SDS-PAGE gel.

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[2] To whom requests for reprints should be addressed, at Department of Biochemistry, Meharry Medical College, 1005 D. B. Todd Boulevard, Nashville, TN 37208. Fax: (615) 327-6442; E-mail: jochieng@mail.mmc.edu.

[3] The abbreviations used are: ECM, extracellular matrix; TDG, thiodigalactoside; CRD, carbohydrate recognition domain.

The cell pellets were lysed in lysis buffer in the presence of protease inhibitors, and the membrane fractions were subjected to SDS-PAGE. The gels were blotted onto nitrocellulose and incubated with avidin-peroxidase followed by chemiluminescence reagents as described previously (7).

Effect of Fetuin on Intracellular Calcium Levels in Breast Cancer Cell Lines. Cells were trypsinized and washed in Krebs-Ringer-HEPES buffer (118.5 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 2.54 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, and 0.03 mM EDTA). They were then loaded with 4 μM of fura-2/AM in the same buffer for 45 min. At the end of the incubation, the cells were washed and placed in a cuvette with stirrer, and fluorescence measurements were made using a SPEX dual wavelength (AR-CM) fluorometer. After about 100 s of stabilization, fetuin was added to a final concentration of 0.25% and changes in intracellular calcium ion concentrations were monitored.

Effects of Calcium Ionophore A23187 and Thapsigargin in the Rapid Release of Galectin-3 from Breast Carcinoma Cells. To determine whether agents that increase intracellular calcium could induce the rapid release of galectin-3, the assay was done in the absence or presence of 5 μM of A23187 in serumless DMEM/F12 containing 1 mM $CaCl_2$. The assay was also done in the presence of 5 μM of A23187 and 0.25% fetuin. Thapsigargin (1 μg/ml), a known inhibitor of the endoplasmic reticulum Ca^{2+} ATPase was also tested in the assay by itself and in the presence of 0.25% fetuin.

Role for the Released Galectin-3 in Cell Spreading and Adhesion. We have previously demonstrated that galectin-3-expressing cells adhere and spread rapidly to ECM proteins compared with galectin-3 null-expressing cells (7, 16). In all of these assays, however, medium containing 10% serum was used. We, therefore, questioned whether galectin-3 release by fetuin was sufficient for this rapid adhesion and spreading. The cells were trypsinized and washed in serumless medium as described above. They were then allowed to adhere to tissue culture microtiter wells in the presence or absence of 0.25% fetuin in serumless DMEM/F12 containing Ca^{2+} ions. The adhesion assay was also done in the presence of 0.25% fetuin and 100 mM TDG. As a negative control (lack of rapid adhesion and spreading), BT-549 cells that do not express galectin-3 were also allowed to adhere in the presence of 0.25% fetuin. The cells were allowed to adhere for 30 min and then were photographed by a digital camera, and the images analyzed by adobe photoshop.

Adhesion of Breast Carcinoma Cells to Elastin in the Absence and Presence of Fetuin. To further implicate galectin-3 in cell-to-ECM interactions, we questioned whether the fetuin-induced rapid release of the lectin from the cells could ligate galectin-3 producing cells to elastin. Elastin (40 mg) was treated with anhydrous hydrazine and 1% hydrazine sulfate for 5 h at 80°C until all of the elastin was dissolved. The solution was then diluted with PBS to a final concentration of 2 mg/ml. The wells of a microtiter plate were then coated with the solubilized elastin for 1 h at 37°C. Nonspecific sites were blocked with 2% BSA, and cells were added to the wells in DMEM/F12 serumless medium without divalent ions and with or without 0.25% Fetuin. The cells were allowed to adhere for 12 h, and the nonadherent cells were washed off in the serumless medium. The adhered cells were photographed, and the number of cells adhered were estimated by the methylene blue assay (17).

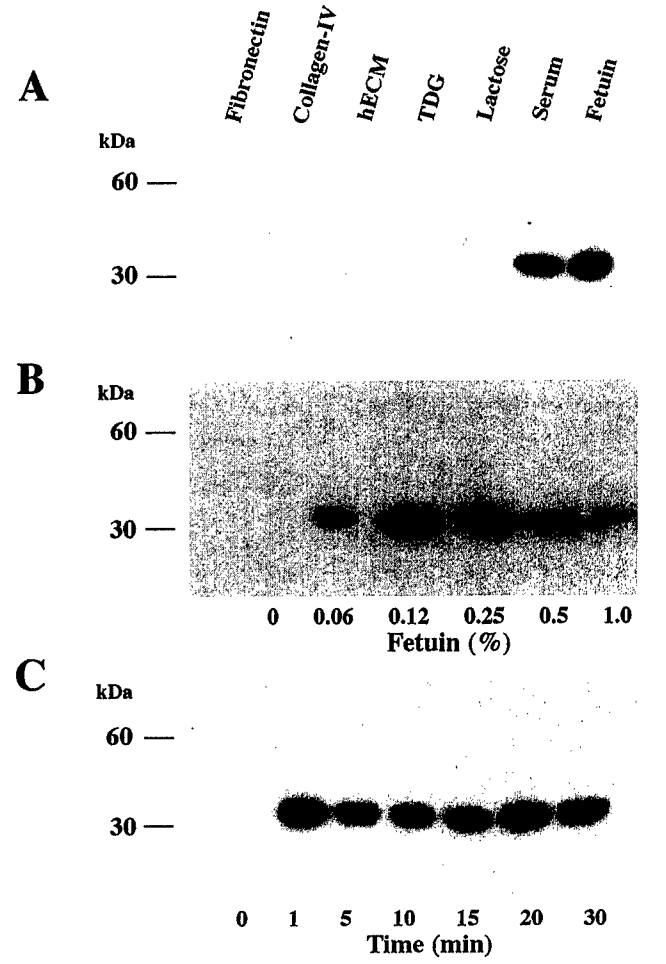

Fig. 1. *Fetuin-mediated release of galectin-3 from MDA-MB-435 breast carcinoma cells into the medium.* In *A*, the cells (500,000 cells/tube) were exposed to the ECM proteins, β galactoside sugars, serum, and fetuin in serumless DMEM/F12 for 10 min. and released galectin-3, assayed by Western blot. In *B*, the cells were exposed to various concentrations of fetuin for 10 min and galectin-3 was assayed. In *C*, the cells were exposed to 0.25% fetuin for the stated time points, and galectin-3 was assayed. *kDa*, M_r in thousands.

RESULTS

Serumless medium in our hands consistently fail to show secreted galectin-3, at least when the blots were exposed to X-ray films in less than 10 min, as we routinely did in this report. As shown in Fig. 1*A*, only fetuin and DMEM/F12 medium supplemented with 10% fetal bovine serum (complete) was able to trigger the release of galectin-3 into the medium. Fetuin was used at a concentration of 0.25% (2.5 mg/ml), which is comparable to the concentration of fetuin in the complete DMEM/F12 medium. From the level of galectin-3 released in both cases, the data suggest that fetuin was the ingredient in DMEM/F12-10% FBS that was responsible for the release of the lectin. Galectin-3 was also released from MDA-MB-435 and 11-9-1-4 by human fetuin, α 2HS glycoprotein (2 mg/ml) (data not shown). It is evident from Fig. 1*B* that the release of galectin-3 is dependent on the dose of fetuin used. Whereas the concentration of fetuin in the fetal blood can be as high as 20 mg/ml, the level drops to approximately 0.6 mg/ml in the adult (18). According to the dose-response data, fetuin levels in both fetus and adult are able to induce the release of galectin-3. The present data suggest that one possible function of fetuin *in vivo* is to trigger the release of intracellular galectin-3, at least in tumor cells. The induction of galectin-3 release by fetuin is very rapid, taking place within 1 min of exposure of cells to fetuin (Fig. 1*C*). Prechilling the cells at 4°C prior to adding fetuin did not affect the release of galectin-3 (data not shown), which demonstrates that the release was not affected by temperature. Inclusion of methylamine or propylamine in the fetuin medium did not change the level of galectin-3 released (data not shown), which implies that the pathway is independent of the endocytosis/exocytosis (4).

The Source of Galectin-3 Released into the Medium. It can be argued that galectin-3 that is rapidly secreted into the medium on fetuin stimulation is cell-surface bound and is not from the intracellular milieu. We, therefore, biotynilated the surface proteins of the breast carcinoma BT-549 clone 11-9-1-4 cells. As evidenced in Fig. 2, all of the label was retained in the cell membrane (Fig. 2, *Lanes 1* and *2*). The conditioned medium in neither the control (Fig. 2, *Lane 3*) nor the fetuin-treated (Fig. 2, *Lane 4*) tubes revealed any protein band after the normal 1–10-min exposure of X-ray film to the immunoblot membrane. Overnight exposure of the film revealed faint bands in

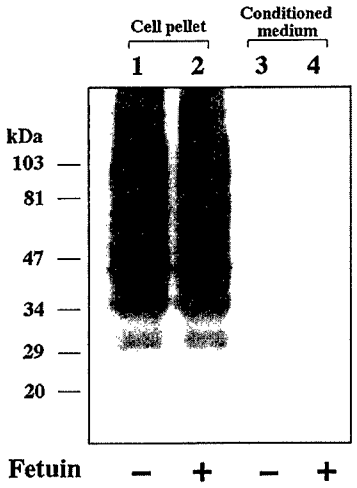

Fig. 2. *Cell surface is not the source of released galectin-3.* The cells (11-9-1-4) were biotynilated and washed, and the cells were incubated without or with fetuin in serumless medium for 30 min. Aliquots of the cell pellet fraction and conditioned medium were run through SDS-PAGE, transferred to immobilon, and the membrane incubated with avidin-peroxidase and chemiluminescent reagents and exposed to X-ray film as described in "Materials and Methods." All of the label was retained in the cell pellet fraction. *kDa*, M_r in thousands.

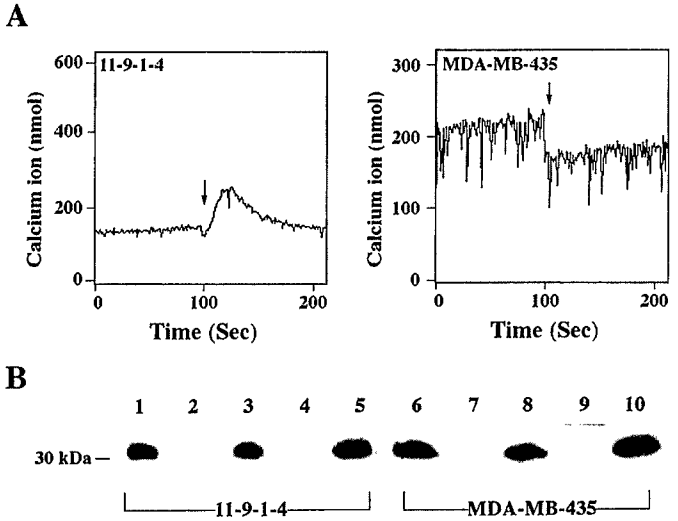

Fig. 3. *Intracellular calcium ion concentration changes and release of galectin-3.* In *A*, intracellular calcium ion changes were determined after the addition of 0.25% fetuin (*arrow*) to fura-2/AM-loaded breast carcinoma cells. The experiment was repeated three times with similar results. In *B*, 11-9-1-4 and MDA-MB-435, were each treated with the following reagents in serumless DMEM/F12 containing 1 mM Ca^{2+}, and galectin-3 was assayed as described: 0.25% fetuin (*Lanes 1* and *6*); calcium ionophore A23187 (5 μM) (*Lanes 2* and *7*); a combination of A23187 and fetuin (*Lanes 3* and *8*); thapsigargin (1 μg/ml) (*Lanes 4* and *9*); and a combination of thapsigargin and fetuin (*Lanes 5* and *10*). *kDa*, M_r in thousands.

both the control and fetuin-treated conditioned medium. Moreover, all of these bands were above M_r 40,000 (data not shown).

Intracellular Calcium Ion Concentration in Fetuin-induced Galectin-3 Release. To investigate the mechanism(s) by which fetuin affects the release of galectin-3, we analyzed intracellular calcium concentration in the breast carcinoma cells before and after the addition of fetuin. Increases in intracellular calcium ion concentration have been shown to stimulate galectin-3 secretion (4, 12). As can be seen in Fig. 3*A*, fetuin raised the intracellular calcium in 11-9-1-4 transiently by about 30%. However, in the MDA-MB-435 cell line, the addition of fetuin actually reduced intracellular calcium levels (Fig. 3*A*). We also challenged the cells with agents that are known to increase intracellular Ca^{2+} ion concentration in the absence and presence of 0.25% fetuin. Galectin-3 was released from both 11-9-1-4 and MDA-MB-435 carcinoma cells in the presence of fetuin (Fig. 3*B*, *Lanes 1* and *6*, respectively). The inclusion of A23187 in serumless medium (Fig. 3*B*, *Lanes 2* and *7*) failed to release galectin-3. The inclusion of A23187/fetuin (Fig. 3*B*, *Lanes 3* and *8*) in the serumless medium triggered the release of galectin-3 in levels comparable with controls. Similarly, thapsigargin, the inhibitor of endoplasmic reticulum ATPase, by itself failed to trigger the release of galectin-3 (*Lanes 4* and *9*) and did not enhance the release in the presence of fetuin (*Lanes 5* and *10*). Taken together, the data suggest that fetuin-induced release of galectin-3 is independent of changes in intracellular calcium.

Rapid Cell Spreading and Adhesion Modulated by Galectin-3 Released from Cells by Fetuin. We show here that the 11-9-1-4 breast carcinoma cells spread and adhere to microtiter wells very rapidly in serumless medium containing fetuin (Fig. 4*A*) compared with adhesion in the absence of fetuin (Fig. 4*B*). The rapid adhesion and spreading in the presence of fetuin was slowed down considerably in the presence of 100 mM TDG (Fig. 4*C*). The parental BT-549 cells that do not express galectin-3 failed to spread and adhere in the presence of fetuin (Fig. 4*D*), requiring an overnight incubation to display cell spreading. The data demonstrate that fast spreading and adhesion to tissue culture plates requires intracellular galectin-3 released rapidly by fetuin. The failure of parental BT-549 to spread and adhere quickly suggests that galectin-3 and not galectin-1 is the relevant lectin. The parental BT-549 cells express high levels of galectin-1 (16).

Breast Carcinoma Cells Adhere to Elastin in the Presence of Fetuin. We have previously demonstrated that the interaction of breast carcinoma cells with elastin is heavily dependent on galectin-3 expression (8). In fact galectin-3 binds specifically to elastin and is associated with tropoelastin in breast carcinoma cells, which suggests that this interaction is physiologically relevant (8). The BT-549 (galectin-3 null-expressing cells) adhered poorly to elastin in the absence

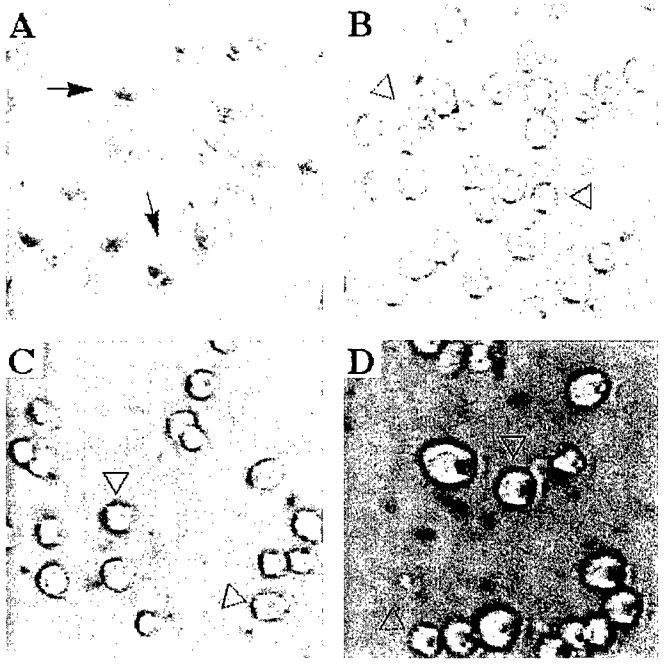

Fig. 4. *Control of rapid cell spreading and adhesion by fetuin.* The 11-9-1-4 cells (galectin-3 expressing) were plated in the wells of a microtiter plate in the presence (*A*) or absence (*B*) of 0.25% fetuin in serumless DMEM/F12 containing 1 mM Ca^{2+}. The cells were also plated in the presence of both fetuin and 100 mM TDG (*C*). As a control, BT-549 (no galectin-3) were plated in the presence of fetuin (*D*). The cells were allowed to adhere for 30 min. and then photographed by a digital camera (phase contrast).

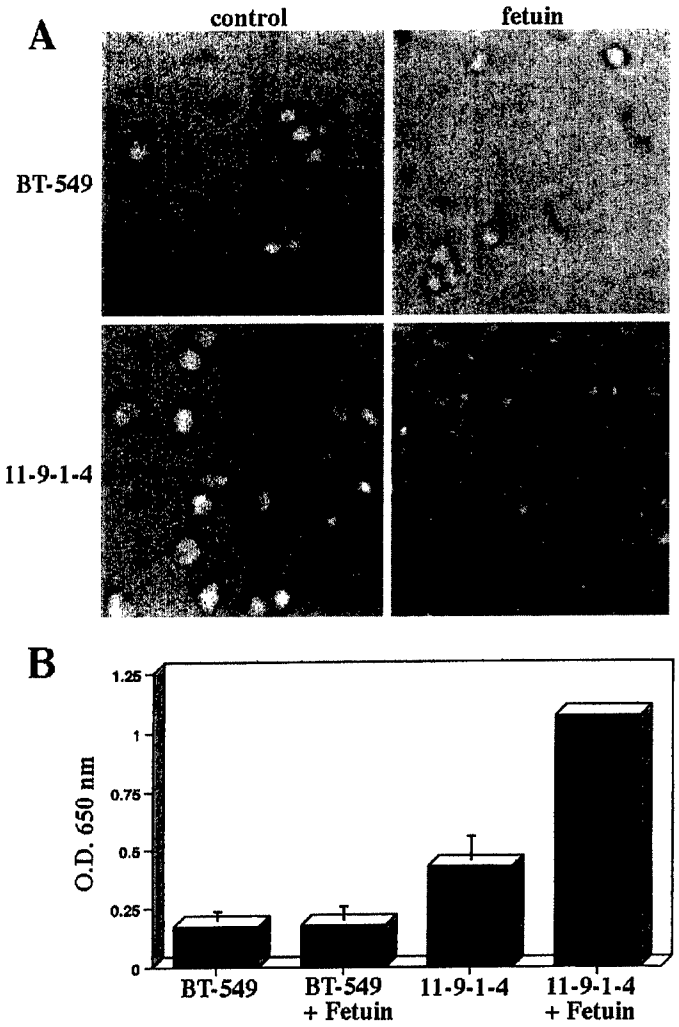

Fig. 5. *Ligation of breast carcinoma cells to elastin.* In *A*, the wells of a microtiter plate were coated with hydrazine-solubilized elastin. After blocking nonspecific sites with 2% BSA, the cells (5×10^4 cells/well) were plated in quadruplicates and were allowed to adhere to the wells in serumless DMEM/F12 medium without divalent ions and without (control) or with 0.25% fetuin and for at least 12 h at 37°C. The nonadherent cells were washed twice and the adherent cells were photographed. In *B*, after photographing the cells, they were fixed in methanol and the cell number determined. *Columns*, represent average number of cells/well. *O.D.*, absorbance.

(control) or presence of 0.25% fetuin as was expected (Fig. 5, *A* and *B*). The 11-9-1-4 cells (galectin-3 expressing), on the other hand, adhered very well to elastin in the presence of fetuin. The interaction was significantly better when compared with adhesion in the absence of fetuin (Fig. 5, *A* and *B*). The data suggest that galectin-3 released by fetuin may be directly used to ligate the breast carcinoma cells to elastin-rich tissues such as the lungs.

DISCUSSION

In this report, we have demonstrated that fetuin, a serum glycoprotein, is capable of eliciting the rapid release of galectin-3 from breast carcinoma cells in a novel fashion. The data address a fundamental problem in biology, namely the role of fetuin and galectin-3 in cell growth regulation. The elucidation of the nonclassical pathway of secretion of galectin-3 is critical in order to understand its precise physiological role in cell-to-ECM interactions. Galectins, particularly galectin-1 and -3, have long been suspected of regulating the adhesion of a wide variety of cell types to ECM (19). We have previously demonstrated that galectin-3 plays a crucial role in the plating and cloning efficiencies of breast carcinoma cells. The cells that express galectin-3 interact more efficiently with substrata such as laminin, collagen IV, elastin, and soft agar compared with galectin-3 null-expressing cells (7, 8, 20). It is well documented that the galectin-3 gene is involved in tumorigenesis (6, 21, 22) and metastasis particularly of the breast and colon carcinomas (16, 23). More recently, it was reported that the sera of patients with breast cancer, gastrointestinal cancer, lung cancer, ovarian cancer, melanoma, and non-Hodgkin's lymphoma had significantly higher levels of galectin-3, compared with sera of normal subjects (24). Moreover, galectin-3 concentrations in sera from patients with metastatic disease were higher than in sera from patients with localized tumors (24). This study suggests that circulating galectin-3 plays a role in tumor progression and that fetuin could be a player in the secretion of galectin-3 into the sera.

The rapid release of galectin-3 on contact of the cells with fetuin and not other ECM proteins is interesting because fetuin and its desialylated form (asialofetuin) interact strongly with galectin-3 via the CRD of the lectin. Normally galectin-3 is expressed on the cell surface presumably bound via its CRD to glycans containing polylactosamine residues such as lysosomal-associated membrane proteins (25). Galectin-3 molecules can also interact with each other via their NH_2-domains, freeing extra CRD domains to interact with other glycoproteins such as laminins in ECM. We, therefore, suspected that the interaction of cells with glycans such as laminin and collagen IV, which have polylactosamine residues, may trigger the release of more galectin-3 from intracellular stores. The present data demonstrate that this is not the case for any of the glycans examined except fetuin. It is, therefore, apparent that the pathway by which fetuin triggers the release of galectin-3 from the intracellular stores is novel. Previous studies demonstrated clearly that serum is essential for galectin-3 secretion. Apart from fetuin, which is the major serum protein in bovine fetal blood, serum contains numerous proteins. In as much as the present study does not rule out all the other serum proteins in the process of galectin-3 release from intracellular stores, fetuin appears to be the critical factor in serum for the externalization of galectin-3 and hopefully other members of the family.

There are a number of models that have been suggested for galectin-3 secretion. For example, it has been proposed that before secretion, galectin-3 accumulates at sites on the cytoplasmic side of plasma membranes (12–14). This step of accumulation is rate limiting and can be up-regulated by heat shock and calcium ionophores (4, 13, 26, 27). The next step in galectin-3 secretion appears to be evagination of plasma membrane, a process that requires NH_2-terminal domains of the protein (15). Finally, the process consists of the pinching off of evaginating plasma membrane domains and the release of galectin-3 from the externalized vesicles. However, as has been noted by others, some galectin-3 molecules may be released from plasma membrane domains directly into the extracellular medium (15). It is this pathway that appears to be supported by our data, because we are defining a process that takes place extremely rapidly (within minutes) and is mediated by fetuin. The molecular mechanisms of this pathway may involve other proteins such as chaperons. Our data clearly demonstrate that galectin-3 is from the cytoplasmic domains and not from the cell surface. The fetuin may induce the secretion of galectin-3/chaperon complex, thereby modulating the last stages of the externalization process and not the rate-limiting step.

On the basis of the data, we propose that the released galectin-3 is immediately recruited to modulate cell spreading and adhesion to the substratum. Galectin-3 may do this by interacting with and activating cell surface adhesion molecules and cytoskeleton elements via its CRD domains because this interaction is abrogated by TDG. Cells that lack galectin-3 but express galectin-1, such as BT-549 breast carci-

noma, lack this rapid cellular adhesion and spreading, as observed previously (7). Interestingly, fetuin has been implicated in cell spreading, stretching, and adhesion in other cell systems (18). The rapid cellular adhesion and spreading that is catalyzed by fetuin and galectin-3 may well explain cell-growth-promoting activities of fetuin (18). The cells that have the capacity to adhere and spread quickly to substrata obviously will have a growth advantage over those that spread and adhere more slowly. We recently demonstrated that the interaction of breast carcinoma cells with elastin could be directly linked to galectin-3 expression because exogenously supplied galectin-3 was able to ligate these cells to elastin. Galectin-3-expressing cells interacted well and proliferated on elastin fibers, but only in the presence of complete medium containing serum (8). We now show that fetuin-mediated release of galectin-3 in serumless DMEM/F12 is sufficient to ligate galectin-3-expressing cells to elastin whereas galectin-3 null-expressing cells are not ligated. The ligation occurred over a 12-h period, because a critical galectin-3 concentration has to be achieved for the adhesion to occur.

It can be argued that the fetuin-induced galectin-3 release is not necessary for *in vivo* cell growth and differentiation, because fetuin-deficient mice are fertile and mature normally (28). This suggests that there are other proteins or growth factors apart from fetuin that may trigger the rapid release of galectin-3. Alternatively, rapid release of galectin-3 may be relevant only in tumor cells, in which it confers a growth advantage. Similarly, galectin-3-null mutant mice are viable with no abnormalities (29). In this case, other members of the galectin family, such as galectin-5, are likely to substitute for galectin-3. Decrease or lack of tumorigenicity or metastatic potential in either fetuin- or galectin-3-deficient mice would be an interesting observation.

In summary, fetuin can induce a very rapid release of galectin-3 from breast carcinoma cells. This release takes place within 1 min and is necessary for the activation and modulation of cell surface receptors for ECM proteins. The released galectin-3 can also be used to ligate breast carcinoma cells to elastin rich tissues such as lungs during the metastatic dissemination of breast cancer. The novel pathway by which galectin-3 is released is independent of changes in intracellular calcium and temperature. The galectin-3 is most likely released from vesicles close to the plasma membrane. The release is not influenced by factors that normally affect the exocytosis or endocytosis pathways. The present data suggest the mechanism(s) by which fetuin may modulate the cellular adhesion and growth of breast epithelial cells *in vitro* and *in vivo*.

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REFERENCES

1. Barondes, S. H., Castronovo, V., Cooper, D. N., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K-I., Leffler, H., Liu, F-T., Lotan, R., Mercurio, A. M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P. W., Rinin, J. M., and Wang, J. L. Galectins—a family of animal-galactoside-binding lectins. Cell, *76:* 597–598, 1994.
2. Perillo, N. L., Marcus, M. E., and Baum, L. G. Galectins: versatile modulators of cell adhesion, cell proliferation and cell death. J. Mol. Med., *76:* 402–412, 1998.
3. Leffler, H. Introduction to galectins. Trends Glycosci. Glycotechnol., *45:* 9–19, 1997.
4. Sato, S., Burdett, I., and Hughes, R. C. Secretion of the baby hamster 30 kDa galactoside-binding lectin from polarized and non-polarized cells: a pathway independent of the endoplasmic reticulum-golgi complex. Exp. Cell Res., *207:* 8–18, 1993.
5. Inohara, H., Akahani, S., Koths, K., and Raz, A. Interactions between galectin-3 and Mac-2 binding protein mediate cell-cell adhesion. Cancer Res., *56:* 4530–4534, 1996.
6. Raz, A., and Lotan, R. Lectin-like activities associated with human and murine neoplastic cells. Cancer Res., *41:* 3642–3647, 1981.
7. Warfield, P. R., Nangia-Makker, P., Raz, A., and Ochieng, J. Adhesion of human breast carcinoma to extracellular matrix proteins is modulated by galectin-3. Invasion Metastasis, *17:* 101–112, 1997.
8. Ochieng, J., Warfield, P., Green-Jarvis, B., and Fentie, I. Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. J. Cell. Biochem., *75:* 505–514, 1999.
9. Rubartelli, A., Bajetto, A., Allavena, G., Wollman, E. and Sitia, R. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. J. Biol. Chem., *267:* 24161–24164, 1992.
10. Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. A novel secretory pathway for interleukin-1-β, a protein lacking a signal sequence. EMBO J., *9:* 1503–1510, 1990.
11. Yu, Z. X., Biro, S., Fu, Y. M., Sanchez, J., Smale, G., Sasse, J., Ferrans, V. J., and Casscells W. Localization of basic fibroblasts growth factor in bovine endothelial cells: immuno-histochemical and biochemical studies. Exp. Cell Res., *204:* 247–259, 1993.
12. Hughes, R. C. Secretion of galectin family of mammalian carbohydrate-binding proteins. Biochim. Biophys. Acta, *1473:* 172–185, 1999.
13. Menon, R. P., and Hughes, R. C. Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-golgi complex. Eur. J. Biochem., *264:* 569–576, 1999.
14. Gong, H. C., Honjo, Y., Nangia-Makker, P., Hogan, V., Mazurak, N., Bresalier, R. S., and Raz, A. The NH_2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells. Cancer Res., *59:* 6239–6245, 1999.
15. Hughes, R. C. The galectin family of mammalian carbohydrate-binding molecules. Biochem. Soc. Trans., *25:* 1194–1198, 1997.
16. Nangia-Makker, P., Thompson, E. W., Hogan, C., Ochieng, J., and Raz, A. Induction of tumorigenicity by galectin-3 in a non-tumorigenic human breast carcinoma cell line. Int. J. Oncol., *7:* 1079–1087, 1995.
17. Oliver, M. H., Harrison, N. K., Bishop, J. E., Cole, P. J., and Laurent, G. J. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell Sci., *92:* 513–518, 1989.
18. Nie, Z. Fetuin: its enigmatic property of growth promotion. Am. J. Physiol., *263:* C551–C562, 1992.
19. Barondes, S. H., Cooper, D. N., Gitt, M. A., and Leffler, H. Galectins: structure and function of a large family of animal lectins. J. Biol. Chem., *269:* 20807–20810, 1994.
20. Raz, A., Zhu, D. G., Hogan, V., Shah, N., Raz, T., Karkash, R., Pazerini, G., and Carmi, P. Evidence for the role of 34 kDa galactoside-binding lectin in transformation and metastasis. Int. J. Cancer, *46:* 871–877, 1990.
21. Lotan, R., Lotan, D., and Raz, A. Inhibition of tumor cell colony formation in culture by a monoclonal antibody to endogenous lectins. Cancer Res., *45:* 4349–4353, 1985.
22. Raz, A., Meromsky, L., Carmi, P., Karkash, R., Lotan, D., and Lotan, R. Monoclonal antibodies to endogenous galactose-specific tumor cell lectins. EMBO J., *3:* 2979–2983, 1984.
23. Bressalier, R. S., Mazurek, N., Sternberg, L. R., Byrd, J. C., Yunker, C. K., Nangia-Makker, P., and Raz, A. Metastasis of human colon cancer is altered by modifying expression of the β-galactoside-binding protein galectin-3. Gastroenterology, *115:* 287–296, 1998.
24. Iurisci, I., Tinari, N., Natoli, C., Angelucci, D., Cianchetti, E., and Iacobelli, S. Concentrations of galectin-3 in sera of normal controls and cancer patients. Clin. Cancer Res., *6:* 1389–1393, 2000.
25. Do, K. Y., Smith, D. F., and Cummings, R. D. LAMP-1 in CHO cells is a primary carrier of poly-*N*-acetyllactosamine chains and is bound preferentially by a mammalian S-type lectin. Biochem. Biophys. Res. Commun., *173:* 1123–1128, 1990.
26. Mehul, B., and Hughes, R. C. Regulation of secretion and surface expression of Mac-2, a galactoside-binding protein of macrophages. J. Biol. Chem., *269:* 4424–4430, 1994.
27. Mehul, B. and Hughes, R. C. Plasma membrane targeting, vesicular budding and release of galectin-3 from the cytoplasm of mammalian cells during secretion. J. Cell Sci., *101:* 1169–1178, 1997.
28. Jahnen-Dechent, W., Schinke, T., Trindl, A., Muller-Esterl, W., Sablitzky, F., Kaiser, S., and Blessing, M. Cloning and targeted deletion of the mouse fetuin gene. J. Biol. Chem., *272:* 31496–31503, 1997.
29. Colnot, C., Fowlis, D., Ripoche, M. A., Bouchaert, I., and Poirier, F. Embryonic implantation in galectin 1/galectin 3 double mutant mice. Dev. Dyn., *211:* 306–313, 1998.